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Genetic analysis of the outer surface protein C gene of Lyme disease spirochaetes (*Borrelia burgdorferi sensu lato*) isolated from rodents in Taiwan

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The outer surface protein C gene (*ospC*) of Lyme disease spirochaetes (*Borrelia burgdorferi sensu lato*) was analysed for the first time in Taiwan. The genetic identities of these Taiwan isolates (TWKM1–7) were determined by restriction fragment length polymorphism (RFLP) analysis and sequence similarities of the PCR-amplified *ospC* gene amplicons. After cleavage by nuclease *Dra*I, differential fragment patterns of PCR-amplified *ospC* DNA in relation to different genospecies of Lyme disease spirochaetes were observed and all of these Taiwan isolates were genetically affiliated to the genospecies of *B. burgdorferi sensu stricto*. The phylogenetic analysis on the sequence similarity of these Taiwan isolates revealed a highly homogeneous genotype, ranging from 99.3% to 100%, within the genospecies of *B. burgdorferi sensu stricto* and was distinguished from other genospecies of *Borrelia* isolates. The sequence similarity analysis also revealed the high sequence variability of the *ospC* gene among *Borrelia* strains that belong to the same genospecies but were isolated from different biological and geographical sources. Thus, these results provide the first investigation on the genetic identity of the *ospC* gene of these Taiwan isolates and show that these Taiwan isolates were closely related genetically to the genospecies of *B. burgdorferi sensu stricto*.

Introduction

Lyme disease is an emerging tick-borne spirochaetal infection [1] that can cause multisystem human illness with varying degrees of clinical symptoms among infected persons, ranging from a relatively benign skin lesion to severe arthritic, neurological and cardiac manifestations [2, 3]. The aetiological agent of Lyme disease, *Borrelia burgdorferi sensu lato*, is transmitted mainly by ticks of the *Ixodes ricinus* complex in North America and Europe [4, 5] and by *I. persulcatus* and *I. ovatus* ticks in the countries of Far East Asia [6–8]. Although a human case of Lyme disease had been reported in Taiwan [9] and *Borrelia* spirochaetes were also isolated from rodents in the Taiwan area [10], the genetic diversity of spirochaetes as well as the tick vectors responsible for transmission in Taiwan need to be established.

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The heterogeneity of molecular and immunological characteristics among isolates of *B. burgdorferi sensu lato* from different geographical and biological origins has been demonstrated previously [11–18]. On the basis of immunoreactivity with *B. burgdorferi*-specific monoclonal antibodies (MAbs), plasmid profiles and the clinical manifestations of the patient, the causative agents of Lyme disease can be classified into three major genospecies – *B. burgdorferi sensu stricto*, *B. garinii* and *B. afzelii* (group VS461) [19, 20]. Furthermore, genomic analysis among *Borrelia* isolates by PCR-restriction fragment length polymorphism (RFLP) analysis and sequence similarity of a specific target gene has been shown to be useful for species identification and genomic typing of *Borrelia* isolates from various biological and geographical sources [21–24].

The outer surface protein C gene (*ospC*) of *B. burgdorferi sensu lato* is located on a 27-kb circular plasmid [25] and is highly heterogeneous with the nucleotide sequences differing among isolates of different species of *Borrelia* [26, 27]. Strain diversity

as well as the genetic heterogeneity can be distinguished among different *Borrelia* isolates by their distinct RFLP types [28]. Thus, the objective of the present study was to characterise the genetic identity of Taiwan isolates by their differential restriction fragment patterns and sequence similarity of the PCR-amplified *ospC* gene amplicons.

Materials and methods

Spirochaete strains and in-vitro cultivation

Seventeen *Borrelia* isolates belonging to the three major genospecies of *B. burgdorferi sensu lato* were used for PCR, RFLP and phylogenetic analysis (Table 1). Spirochaetes were cultured at 34°C in a humidified incubator (Nuaire, Plymouth, MN, USA) with CO_2 5% in air and maintained in BSK-H medium (catalogue no. B3528; Sigma) supplemented with rabbit serum (catalogue no. R7136; Sigma) 6% as described previously [10]. All cultures were examined weekly for optimal growth of spirochaetes by dark-field microscopy (model BX-60, Olympus, Tokyo, Japan).

Preparation of spirochaete DNA

Total genomic DNA was extracted from all *Borrelia* isolates as described previously [29]. Briefly, (3-ml) samples of cultured spirochaetes grown to a density of *c.* 2×10^8 cells/ml of medium were centrifuged for 10 min at 12 000 *g* to pellet the spirochaetes. The pellets were washed twice with phosphate-buffered saline (PBS; pH 7.2) containing 5 mM $MgCl_2$, resuspended in 150 μl of distilled water and boiled for 10 min. After centrifugation at 10 000 *g* for 10 s, the supernate was collected and the DNA concentration was determined spectrophotometrically with a DNA calculator (GeneQuant II; Pharmacia Biotech, Uppsala, Sweden).

Table 1. Strains of *B. burgdorferi sensu lato* analysed

Genospecies and strain no.	Origin		*ospC* gene accession no.
	Biological	Geographical	
B. burgdorferi sensu stricto			
B31	Tick	USA	AF411450
JD 1	Tick	USA	AF416424
N40	Tick	USA	AF416430
CT27985	Tick	USA	AF416428
TB	Tick	USA	AF416431
VS219	Tick	Switzerland	AF416432
CT20004	Tick	France	AF416427
ECM-NY86	Human skin	USA	AF416429
B. garinii			
K48	Tick	Czechoslovakia	AF416425
B. afzelii			
VS461	Tick	Switzerland	AF416426
Taiwan isolates			
TWKM1	Mouse ear	Taiwan	AF411451
TWKM2-7	Mouse ear	Taiwan	AF416418-23

Genetic analysis by PCR and RFLP

DNA samples extracted from the Taiwan isolates and other spirochaetes representative of the three major genospecies of *B. burgdorferi sensu lato* were used as template for PCR amplification of the *ospC* gene DNA. An *ospC*-specific primer set of CF1 (forward) 5′-AAGTGCGATATTAATGAC-3′ and CR2 (reverse) 5′-GATCTTTCTGCCACAACAG-3′ were designed and synthesised by a custom oligonucleotide synthesis service (Gibco BRL, Taipei, Taiwan) as described previously [30]. All PCR reagents and TaqGold DNA polymerase were obtained from the GeneAmp kit and were used as recommended by the supplier (Perkin-Elmer Cetus, Taipei, Taiwan).

Briefly, a total of 20-pmol of the appropriate primer set and various amounts of template DNA were used in each 50-μl reaction mixture. PCR amplification was performed with a Perkin-Elmer Cetus thermocycler (GeneAmp system 9700) and with amplification for 30 cycles of denaturation at 92°C for 30 s, annealing at 41°C for 30 s, and extension at 72°C for 90 s. PCR-amplified DNA products were electrophoresed on agarose 2% gels in TBE buffer and were visualised under UV light after staining with ethidium bromide. The 1-kb plus DNA ladder (catalogue no. 10787-018, Gibco BRL, Taipei, Taiwan) was used as the standard marker for comparison. After purification with a QIAquick PCR purification kit (catalogue no. 28104, Qiagen, Taipei, Taiwan), the purified PCR products were digested with restriction endonuclease *Dra*I (New England Biolabs, Taipei, Taiwan) according to the manufacturer's instructions. The digested DNA fragments were separated by electrophoresis in agarose 3.5% gels (Agarose-1000, Gibco BRL) and visualised under UV light with ethidium bromide staining. DNA ladders of 1-kb plus and 50 bp (10416-014, Gibco BRL) fragments were used as the standard markers for comparison.

Sequence alignments and phylogenetic analysis

The nucleotide sequences of the *ospC* gene amplicon of the *Borrelia* isolates used in this study were sequenced by a dye-deoxy terminator reaction method with the bigdye terminator-Taq cycle sequencing kit and an ABI Prism 377-96 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The determined sequences were aligned and a similarity matrix and neighbour-joining phylogenetic tree were constructed with the DNASTAR program and the CLUSTAL V software package [31, 32].

Nucleotide sequence accession numbers

The nucleotide sequences of PCR-amplified *ospC* gene amplicons determined in this study have been registered and assigned the following GenBank accession numbers: strains B31 (AF411450), TWKM1 (AF411

451), TWKM2 (AF416418), TWKM3 (AF416419), TWKM4 (AF416420), TWKM5 (AF416421), TWKM6 (AF416422), TWKM7 (AF416423), JD1 (AF416424), K48 (AF416425), VS461 (AF416426), CT20004 (AF416427), CT27985 (AF416428), ECM-NY86 (AF416429), N40 (AF416430), TB (AF416431) and VS219 (AF416432).

Results

The *ospC* gene was amplified by PCR to generate the *ospC* DNA of all the Taiwan isolates and another 10 isolates belonging to three major genospecies of Lyme disease spirochaetes. A DNA fragment of *c.* 630 bp was generated and observed in an agarose 2% gel (Fig. 1). These results demonstrate that the *ospC* genes were highly conserved in all the *Borrelia* isolates regardless of their origin and the genospecies of the Lyme disease spirochaetes.

To clarify the genomic identity of these Taiwan isolates, PCR-RFLP analysis of the *ospC* gene amplicon was also performed for further characterisation of *Borrelia* isolates belonging to the three major genospecies of Lyme disease spirochaetes. After cleavage by *Dra*I, the restriction site polymorphism of the *ospC* gene amplicons from the 17 *Borrelia* isolates was investigated by comparing their restriction patterns of digested DNA fragments. All 17 isolates could be classified into four different RFLP patterns (Fig. 2) in relation to three different genospecies of *B. burgdorferi sensu lato*. The restriction fragment pattern of *Dra*I-digested *ospC* gene amplicon of the genospecies *B. garinii* (strain K48) demonstrated a specific pattern (type B) with DNA fragments of *c.* 260, 220

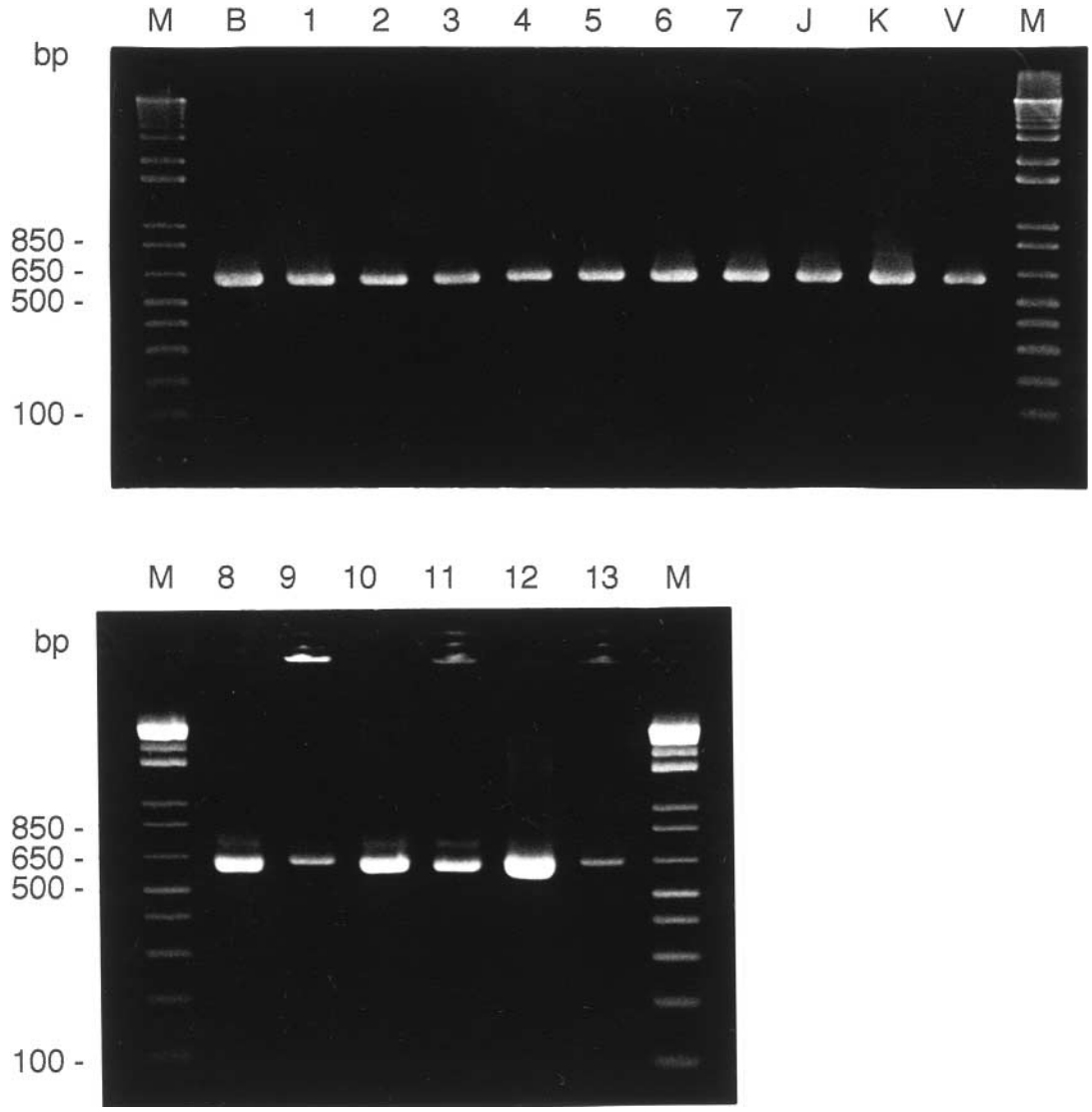

Fig. 1. PCR analysis with a primer set specific for the *ospC* genes of *B. burgdorferi sensu lato*. Lane **B**, isolate B31; lanes **1**–**7** represent the Taiwan isolates of TWKM1–7, respectively; lane **J**, isolate JD 1 of *B. burgdorferi sensu stricto*; lane **K**, isolate K48 of *B. garinii*; lane **V**, isolate VS461 of *B. afzelii*; lanes **8**–**13** represent the *B. burgdorferi sensu stricto* strains CT20004, CT27985, ECM-NY86, N40, TB and VS219, respectively; lane **M**, 1-kb plus DNA ladder (Gibco BRL). The amplification products were electrophoresed on agarose 2% gels (Agarose-LE, USB, Cleveland, OH, USA) and DNA fragments of *c.* 630 bp were visualised under UV light with ethidium bromide staining.

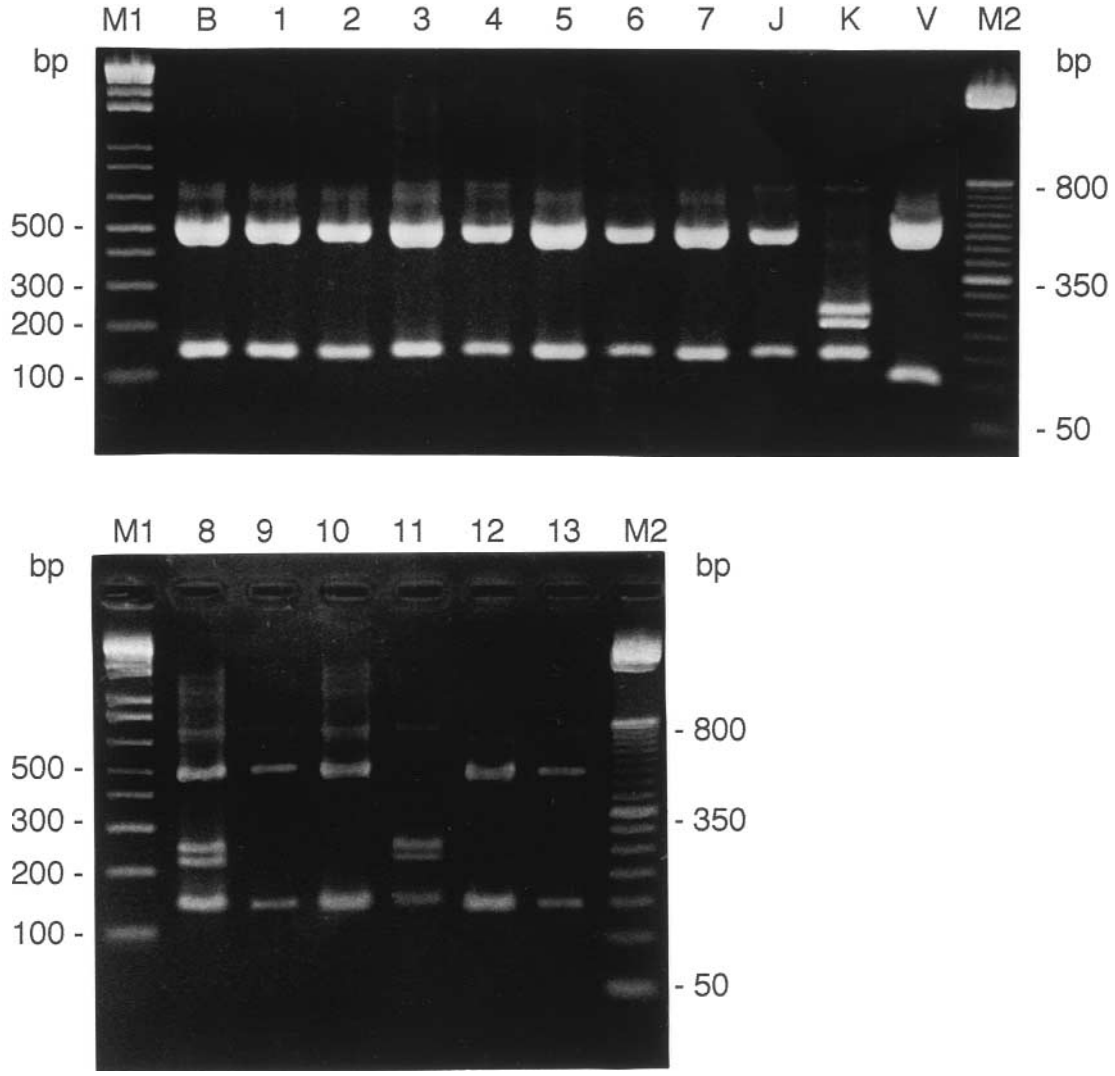

Fig. 2. PCR-RFLP analysis of the restriction patterns of *ospC* genes from Taiwan isolates and isolates of other genospecies of *B. burgdorferi*. The amplification products were further purified by a QIAquick PCR purification kit (Qiagen) and were digested with restriction endonuclease *Dra*I. The digested DNA fragments were electrophoresed on an agarose 3.5% gel (Agarose-1000, Gibco BRL), stained with ethidium bromide and visualised by UV transillumination. Lane **B**, isolate B31; lanes **1**–**7** represent the Taiwan isolates TWKM1–7, respectively; lane **J**, isolate JD 1 of *B. burgdorferi sensu stricto*; lane **K**, isolate K48 of *B. garinii*; lane **V**, isolate VS461 of *B. afzelii*; lanes **8**–**13** represent the *B. burgdorferi sensu stricto* strains CT20004, CT27985, ECM-NY86, N40, TB and VS219, respectively; lanes **M1** and **M2** represent the 1-kb plus and 50-bp DNA ladders (Gibco BRL), respectively.

and 150 bp, and a specific pattern (type C) with DNA fragments of *c.* 510 and 120 bp was also observed with the *ospC* gene amplicon of *B. afzelii* (strain VS461). All seven Taiwan isolates exhibited the same pattern (type AI) as the other six strains of *B. burgdorferi sensu stricto* with DNA fragments of *c.* 480 and 150 bp (Table 2). However, a different restriction fragment pattern (type AII) was observed in the *Dra*I-digested *ospC* amplicons of strains N40 and CT20004 with DNA fragments of *c.* 270, 230 and 150 bp. These results revealed the genetic diversity of *ospC* genes among *Borrelia* isolates from different origins or of different genospecies of *B. burgdorferi sensu lato*. All of these Taiwan isolates were genetically related to the genospecies *B. burgdorferi sensu stricto*.

The sequence similarity of the *ospC* gene was analysed to identify the homogeneity of the Taiwan isolates in relation to the genospecies of *B. burgdorferi sensu lato*. As shown in Table 3, the nucleotide sequences of *ospC* gene amplicons were highly homogeneous and ranged from 99.3 to 100% among Taiwan isolates and the other four *Borrelia* isolates (strains B31, JD1, CT27985 and TB) within the genospecies *B. burgdorferi sensu stricto*. However, the homogeneity of *ospC* nucleotide sequences among the strains N40, ECM-NY86, VS219 and CT20004 ranged from 71.4 to 80.0% in comparison to the genospecies *B. burgdorferi sensu stricto* (Table 3). Furthermore, the *ospC* sequence of strains K48 (*B. garinii*) and VS461 (*B. afzelii*) showed a homogeneity of only 65.4–74.6% and 65.6–77.9%, respectively.

The phylogenetic relationships based on the sequence alignment of *ospC* gene were also analysed to demonstrate the divergence among *Borrelia* isolates investigated in this study. As compared with the aligned sequence of isolate B31, highly variable nucleotide

Table 2. RFLP analysis of restriction patterns of the *ospC* gene from Taiwan isolates and other genospecies of *B. burgdorferi sensu lato*

Genospecies and strain no.	Estimated PCR product (bp)	*Dra*I restriction fragment sizes (bp)	RFLP type
B. burgdorferi sensu stricto			
B31	630	150, 480	AI
JD1	630	150, 480	AI
N40	630	150, 230, 270	AII
CT27985	630	150, 480	AI
TB	630	150, 480	AI
VS219	630	150, 480	AI
CT20004	630	150, 230, 270, 500*	AII
ECM-NY86	630	150, 480	AI
Taiwan isolates			
TWKM1-7	630	150, 480	AI
B. garinii			
K48	630	150, 220, 260	B
B. afzelii			
VS461	630	120, 510	C

*Incompletely digested DNA fragment.

sequences were observed in strains CT20004, VS219, ECM-NY86 and N40, with a sequence divergence of 15–21% within the genospecies *B. burgdorferi sensu stricto* (Fig. 3). However, these sequences can be distinguished from those of strains K48 (*B. garinii*) and VS461 (*B. afzelii*). Furthermore, all the Taiwan isolates were very similar to strains B31, JD1, TB and CT27985 with a sequence divergence $<0.5\%$. These results reveal the heterogeneity of the *ospC* gene among *Borrelia* isolates within the same genospecies as well as between the genospecies of *B. burgdorferi sensu lato* and all of these Taiwan isolates could be verified as belonging to the genospecies *B. burgdorferi sensu stricto*.

Discussion

This report describes the first genomic characterisation and classification of the *ospC* gene among Lyme disease spirochaetes (*B. burgdorferi sensu lato*) isolated in Taiwan. In previous investigations, the protein profiles of these Taiwan isolates were consistent with the major protein bands of other documented strains of Lyme disease spirochaetes and their antigenicity was also verified by their reactivities with MAbs specific for *B. burgdorferi sensu lato* [29]. Although the heterogeneity among major protein bands and the immunoreactivity with *B. burgdorferi*-specific MAbs had been used for the typing or species identification of Lyme disease isolates, the validity of these methods for genospecies identification was not fully satisfied [14, 33]. Thus, genomic analysis based on the *ospC* genes may provide a reliable and useful method for species identification of *Borrelia* spirochaetes that exist in various animal reservoirs and vector ticks of Taiwan.

Although genetic analysis based on the genospecies-specific PCR primers had been recognised as a rapid and definitive assay for species identification of *Borrelia* spirochaetes from various biological and geographical origins [14, 21–24, 33], it was difficult to clarify the genetic diversity among *Borrelia* isolates at the intraspecies level [34]. Moreover, genetic heterogeneity can be determined among *Borrelia* isolates that were previously identified as the same genospecies or atypical strains of spirochaetes [28, 35, 36]. In a previous study, the genetic relationship of these Taiwan isolates was determined to be the same genospecies by their differential reactivities with genospecies-specific PCR primers based on the *ospA* gene of *B. burgdorferi sensu lato* [29]. Results from the present study further clarify the genetic identity of these Taiwan isolates by analysing the *ospC* gene amplicons and all these Taiwan isolates were genetically classified into one subtype (AI) within the genospecies *B. burgdorferi sensu stricto*.

The genetic heterogeneity of *Borrelia* isolates can be classified according to their differential restriction fragment patterns by RFLP analysis of specific target genes [21, 37–39]. Previous reports concluded that RFLP analysis of the *ospC* genes seemed useful for detection of mixed spirochaetal infections and classification of the genospecies of *Borrelia* isolates detected in various biological specimens [25–28, 38]. Results of the present study also demonstrate that the genospecies could be determined among different *Borrelia* isolates by their restriction fragment patterns of the *ospC* gene and reveal two subgroups within the genospecies *B. burgdorferi sensu stricto* (Fig. 2 and Table 2). All the Taiwan isolates were genetically linked to one major subgroup (AI) according to the homogeneity of RFLP pattern within the same genospecies of *Borrelia*. These observations suggest that the genomic identity of *Borrelia* isolates can be determined either interspecies or intraspecies in *B. burgdorferi sensu lato* by analysing the restriction polymorphisms of *Dra*I-digested *ospC* gene amplicons.

The phylogenetic relationships among *Borrelia* isolates can be determined by analysing the sequence similarity of a specific target gene. Sequence analysis of the *ospC* gene among *Borrelia* isolates had been shown to be useful for evaluating the taxonomic relatedness of *B. burgdorferi sensu lato* isolates derived from various geographical and biological sources [25–28]. The phylogenetic analysis of the *ospC* gene sequence of isolates previously identified as the same or atypical strains of *Borrelia* also differentiated new ribotype groups within the same genospecies of Lyme disease spirochaetes [26]. Moreover, nucleotide sequence variation of a target gene may actually represent genetic divergence within the genospecies of *Borrelia* isolates and genetic exchange by lateral transfer of the *ospC* sequence is proposed to be the mechanism responsible for the relatively high levels of *ospC* gene diversity [27, 28]. In this study, the phylogenetic analysis of the *ospC* gene among 17 *Borrelia* isolates

Table 3. Sequence similarity between *ospC* gene sequences from Taiwan isolates and isolates of other genospecies of *Borrelia*[*]

	Sequence similarity (%) to strain no.																
Strain no.	B31	Twkm1	Twkm2	Twkm3	Twkm4	Twkm5	Twkm6	Twkm7	JD-1	K48	VS461	CT20004	CT27985	ECM-NY86	N40	TB	VS219
B31	100.0	99.6	99.8	99.8	100.0	99.6	99.5	99.3	99.3	74.5	77.9	80.0	99.7	76.8	79.3	100.0	71.6
Twkm-1		100.0	99.8	99.3	99.6	99.1	99.6	99.6	99.6	74.1	77.5	79.8	99.6	77.3	79.1	99.5	72.1
Twkm-2			100.0	99.6	99.8	99.1	99.8	99.8	99.8	74.5	77.7	79.8	99.8	77.4	79.3	99.6	71.9
Twkm-3				100.0	99.8	98.9	99.8	99.8	99.8	74.1	77.6	79.7	99.8	77.1	79.0	99.1	71.4
Twkm-4					100.0	99.6	99.5	99.3	99.3	74.5	77.9	80.0	99.7	76.8	79.3	100.0	71.6
Twkm-5						100.0	99.1	99.3	99.3	74.3	77.5	79.8	99.6	77.2	79.3	99.8	71.8
Twkm-6							100.0	99.8	99.8	74.6	77.4	79.4	99.1	76.7	78.7	99.3	71.1
Twkm-7								100.0	100.0	74.6	77.4	79.4	99.3	76.7	78.9	99.3	71.4
JD-1									100.0	74.6	77.4	79.4	99.3	76.7	78.9	99.3	71.4
K48										100.0	77.3	71.1	74.5	70.2	77.0	74.5	65.4
VS461											100.0	73.7	77.7	70.6	76.2	77.8	65.6
CT20004												100.0	79.8	75.8	75.0	79.9	70.1
CT27985													100.0	76.0	79.3	99.8	72.1
ECM-NY86														100.0	69.0	77.6	89.4
N40															100.0	79.4	65.6
TB																100.0	72.0
VS219																	100.0

[*]Strains B31, JD1, CT20004, CT27985, ECM-NY86, N40, TB and VS219, *B burgdorferi sensu stricto*; K48, *B garinii*; VS461, *B. afzelii*.

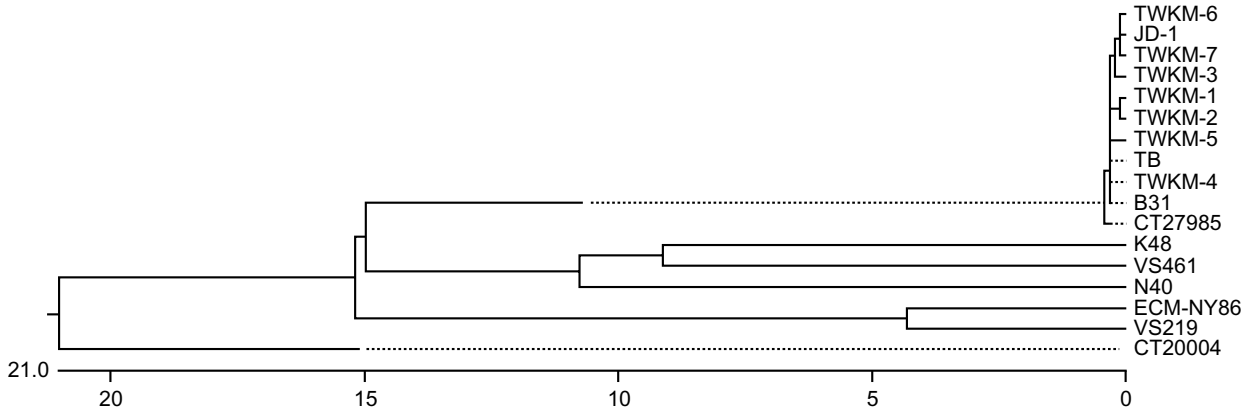

Fig. 3. Phylogenetic tree based on a comparison of the *ospC* gene sequences from 7 Taiwan isolates and 10 strains of *B. burgdorferi sensu lato*. The bar represents the divergence beween sequences of these *Borrelia* isolates.

demonstrated a high sequence heterogeneity between different genospecies and within the same genospecies of *B. burgdorferi sensu stricto* (Fig. 3). Further investigation on the sequence analysis of *ospC* genes of *Borrelia* strains isolated from various reservoir hosts, vector ticks and patients would help to clarify the genetic divergence of *Borrelia* isolates in Taiwan.

In conclusion, this report describes the first identification and characterisation of the *ospC* gene of *Borrelia* spirochaetes isolated in Taiwan. On the basis of their differential RFLP patterns and sequence similarity of their *ospC* genes, all these Taiwan isolates were genetically related to the genospecies of *B. burgdorferi sensu stricto*. Further application of this molecular tool to identify the genetic variability of the *ospC* genes from *Borrelia* isolates from patients, reservoir animals and vector ticks may help to illustrate the significance of the diversity of *Borrelia* spirochaetes in relation to the epidemiological features of human Lyme borreliosis in Taiwan.

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